EXHIBIT 5

IN VIVO DELIVERY OF THERAPEUTIC PROTEINS BY GENETICALLY MODIFIED CELLS: COMPARISON OF ORGANOIDS AND HUMAN SERUM ALBUMIN ALGINATE COATED BEADS

E. SHINYA[1], X. DERVILLEZ[2], F. EDWARDS-LÉVY[3], V. DURET[2], E. BRISSON[1], L. YLISASTIGUI[1], M.C. LÉVY[3], J.H.M. COHEN[2], D. KLATZMANN[1*]

[1]Laboratoire de Biologie et Thérapeutique des Pathologies Immunitaires, UPMC/CNRS ESA 7087, CERVI, Hôpital de la Pitié-Salpêtrière, 83 Bd de l'Hôpital, 75651 Paris Cedex 13 France
Tel.:(+33)1 42 17 74 61 Fax.: (+33) 1 42 17 74 62

[2] Laboratoire d'Immunologie, CHU Robert Debré, 51092 Reims France

[3] Laboratoire de Pharmacotechnie, CNRS URA 492, Faculté de Pharmacie, 51096 Reims, France

* corresponding author

Summary

We have designed a self-assembling multimeric soluble CD4 molecule by inserting the C-terminal fragment of the alpha chain of human C4-Binding protein (C4bpα) at the C-terminal end of human soluble CD4 gene. This CD4-C4bpα fusion protein (sMulti-CD4) and two other reference molecules, a fusion protein of human serum albumin (HSA) and the first two domains of CD4 (HSA-CD4) and monomeric soluble CD4 (sMono-CD4), were delivered *in vivo* by genetically modified 293 cells. These cells were implanted in mice as organoids and also encapsulated in HSA-alginate coated beads. sMulti-CD4 showed an apparent molecular weight of about 300-350 kDa, in accordance with a possible heptamer formula). sMulti-CD4 produced either in cell culture or in vivo in mice appeared a better *in vitro* inhibitor of HIV infection than sMono-CD4. Plasma levels of sMulti-CD4, HSA-CD4, and sMono-CD4 reached approximately 2300, 2700, and 170 ng/ml, respectively, 13 weeks after *in vivo* organoid implantation that had formed tumour at that time. This suggest that the plasma half-life of sMulti-CD4 is much longer than that of sMono-CD4. The 293 xenogenic cells encapsulated in HSA- alginate coated beads remained alive and kept secreting sMono-CD4 or HSA-CD4 continuously at significant level for 18 weeks in nude mice, without tumour formation. When implanted in immuno-competent Balb/c mice, they were rejected In 2-3 weeks after implantation. In contrast, encapsulated BL4 hybridoma cells remained alive and kept secreting BL4 anti-CD4 mAb for at least 4 weeks in Balb/c mice. These results suggest the clinical potential of the C4bp-multimerizing system, which could improve both the biological activity and the poor *in vivo* pharmacokinetic performance of the monomeric functional protein like soluble CD4. These data also show that a systemic delivery of

therapeutic proteins, including immunoglobulins, can be obtained by the *in vivo* implantation of engineered allogeneic cells encapsulated in HSA-alginate coated beads.

Keywords :

HIV/Multivalent/gene therapy

Abbreviations :

mAb: monoclonal antibody, sMono-CD4 : Soluble monomeric CD4, sMulti-CD4 : Soluble multimeric CD4

INTRODUCTION

Somatic gene therapy for delivering therapeutic proteins could be an attractive option for several clinical settings such as haemophilia B [1], growth hormone deficiency [2], mucopolysaccharidosis [3-5] or ß thalassemia [6-11]. Current approaches are often based on the implantation of genetically modified autologous cells. Likewise, genetically modified fibroblasts can be prepared from a skin biopsy, expanded, and re-implanted after their aggregation on fibres. Such « organoids » become vascularized and secrete the recombinant proteins of interest. However, such a 'tailored' therapy, in which the genetic modification as well as the necessary quality control tests need to be performed for each patient, is labour-intensive and costly. So, it might be applicable for the treatment of rare diseases, but not for prevalent diseases such as HIV infection. For the latter settings, the design of strategy that allows the implantation of non-autologous cells would be an alternative approach that is currently under investigation. Indeed, new technologies such as the encapsulation of allogenic cells are under development [12]. They are aimed at protecting the transplanted cells from being rejected by the host immune system, while allowing the secretion of the therapeutic proteins [13, 14].

We aim to develop new therapy for HIV infection based on the secretion of antiviral proteins. We previously reported the long term *in vivo* delivery of a soluble form of the HIV receptor, the CD4 molecule. Mouse fibroblasts were retrovirally transduced with a soluble CD4 gene (sCD4), and reimplanted as organoids in transgenic mice expressing human CD4 and thus tolerant to sCD4 [15]. The sCD4 serum levels obtained were significant but too limited for an expected antiviral effect. This appears primarily due the pharmacokinetic properties of sCD4 that has a very short plasma half-life [16]. We and others have designed CD4-based

recombinant chimeric proteins aimed to increase their anti-HIV properties as well as their half lives [17-21]. CD4-Ig molecules are still under development for clinical use, in association with other antiviral molecules such as *reverse transcriptase* inhibitors or anti-proteases. We have already designed a fusion protein comprising human serum albumin (HSA) and the first two domains of CD4 (HSA-CD4) [20]. This recombinant protein has a long half life in the serum, comparable to that of human serum albumin. We also recently designed a multimeric CD4 fusion protein based on a C-terminal fragment of human C4 binding protein alpha chain (C4bpα), a naturally heptameric protein [22]. CD4-C4bpα should thus be secreted as an heptamer, and such a large and multimeric molecule is expected to have longer half life, better stability *in vivo*, and better anti-HIV activity.

We thus aimed to analyse the feasibility of secreting anti-HIV therapeutic proteins by organoids or cell encapsulation into HSA-alginate coated beads. We generated stable cell lines secreting either sMono-CD4, HSA-CD4 or sMulti-CD4. We then re-implanted these cells as organoids or after encapsulation in HSA-alginate coated beads [23], and monitored the expression of the recombinant proteins in sera of immuno-deficient or immuno-competent mice. Our results indicate that plasma concentrations dramatically depend on the number of secreting cells and also on the half life of the recombinant proteins. Plasma concentrations of HSA-CD4 up to 125µg/ml were achieved. Furthermore, we show that allogenic cells encapsulated in the coated-alginate beads could represent an efficient way to secrete recombinant therapeutic proteins.

These results warrant further developments of this therapeutic strategy for the treatment of HIV infection. Indeed, CD4-based molecules, neutralising antibodies directed

against viruses or against HIV receptors or co-receptors [24] are capable of inhibiting HIV infection and could benefit of these delivery methods.

MATERIALS AND METHODS

Construction of expression plasmids

The 177-base pair C-terminal C4bp α-chain fragment was amplified using the following primers :

5'-GAGACCCCCGAAGGCTGTGA-3'

5'-ATTTCTAGA GAGTTATAGTTCTTTATCCAAAGTGGA-3'

Underlined sequence represents XbaI restriction site. The stop codon is shown in bold characters. Polymerase chain reaction was done as described previously, by using genomic DNA of HepG2 cells as template [22].

The 6.2 kb pST4 plasmid containing the sequence of the four extracellular domains of human CD4 [25] was digested by EcoRI and AvaI. CD4 coding fragment was linked at its 3' end to an AvaI digested oligonucleotide

(5'-**TCGGA**ACAGGTCCTGCTGGAATCCAACATCAAGGTTCTGCCCACATGG-3')

previously linked to the C4bp α-chain fragment. Then, this EcoRI-XbaI fragment was subcloned into the multiple cloning site of pCI plasmid (Promega) to make CD4-C4bpα/pCI. This construct led to the expression of a multimeric covalent molecule referred to as sMulti-CD4.

A coding fragment of sMono-CD4 from pM48-sCD4 [15] or that of HSA-CD4 from pYG365B [20], was also cloned into the multiple cloning site of pCI to make sCD4/pCI and HSA-CD4/pCI respectively (Fig.1A).

Selection of sMulti-CD4, HSA-CD4 or sMono-CD4 secreting 293 clones

Subconfluent 293 cells (ATCC CRL 1573) in a 10 cm dish, which were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, were co-

transfected with 0.5 µg of pMC1neo poly A (Stratagene) and 20 µg of CD4-C4bpα/pCI, HSA-CD4/pCI or sCD4/pCI by standard calcium phosphate co-precipitation method. 48 h later, G418 was added to the medium at 500 µg/ml. After 14 days, neomycin resistant clones were isolated and individually expanded. Secretion of the recombinant protein was then assayed by ELISA.

ELISA procedures

For HSA-CD4, ELISA plates (Maxisorp, Nunc, Denmark) were coated with IgG fraction of a polyclonal rabbit anti-HSA serum (Sigma, dilution 1:2000), blocked with 1 x Power Block° solution (BioGenex, USA), and incubated with samples of culture supernatant or plasma from the organoids-implanted mice, which were diluted with 1 x Power block solution. Leu3a anti-CD4 mAb or Leu3a conjugated with biotin (Becton and Dickinson, dilution 1/125) was then added, followed by the *peroxidase*-linked anti-mouse IgG serum (DAKOPATTS, dilution 1/2000) or streptavidin-*POD* (Boehringer Mannheim, dilution 1/800) respectively. Absorbance at 405 nm was measured after the addition of ABTS solution (BochringerMannheim).

All sMulti-CD4 and sMono-CD4 measurements in culture medium and plasma, were performed using the sCD4 ELISA kit (Boehringer Mannheim)detecting the number of CD4 binding sites for the revealing antibody. Results are thus expressed in ng/ml with respect to a standard curve made with purified recombinant sCD4.

Protein electrophoresis and Western blotting

Electrophoresis was performed in a precast Tris-Glycine 4-12% polyacrylamide gel (Novex, USA). Pre-stained Rainbow markers (Amersham, UK) were used for gel calibration. The culture supernatant of the 293 cells secreting sMulti-CD4, HSA-CD4 or sMono-CD4, or diluted plasma of the organoids implanted nude mice were loaded in reducing (Tris-HCl 63mM, Glycerol 10%, SDS 2%, dithiothreitol (DTT) 50mM) or non-reducing condition (Tris-HCl 63mM, Glycerol 10%, SDS 2%). After electrophoresis, proteins were transferred to a nitrocellulose membrane (0.45-µm pore size, Shleicher & Schueel) at 4°C overnight by electro-transfer using Mini-TransBlot (BioRad). To ensure equivalent protein loading in each lane and correct transfer, bands were made visible by staining with 0.3% Ponceau Red staining. After staining, membranes were rinsed in TBS. Then, the filter was blocked, incubated with a rabbit polyclonal anti-human CD4 serum (K582) [19] for sMulti-CD4 and sMono-CD4, or with a rabbit polyclonal anti-HSA IgG (Sigma) for HSA-CD4, then developed using *peroxidase*-linked anti-rabbit IgG serum (DAKOPATTS) and a chemiluminescence western blot kit (BoehringerMannheim).

HIV-1 viruses and viral infection procedures

P4-CCR5 infection : The derivation and characterisation of the P4-CCR5 cells has been previously described [26]. Cells are grown in Dulbecco's modified Eagles' medium supplemented with 10% Foetal Calf Serum (Gibco), 500 µg/ml G418 and 1 µg/ml puromycin. The P4-CCR5 cells were plated (10^4 cells/well of 96-well plate, 3 x 10^4 cells/well of 48-well plate) one day prior to infection. On the day of the infection, one hundred tissue culture infectious doses of HIV-1_{LAI} strain of HIV-1 (HIV-1_{LAI}, Diagnostics Pasteur, France) were first incubated with several concentrations of sMulti-CD4 or sMono-CD4 for 2 hours. Then

those mixture of HIV-1_{LAI} and sMulti-CD4 or sMono-CD4 are added to the cells for infection in the presence of 20 µg/ml of DEAE Dextran. After 48 hours of infection, medium was removed and the enzymatic detection of β-Gal was performed using β-gal reporter gene assay kit (Boehringer Mannheim).

Organoids formation and implantation

The 293 clones secreting sMulti-CD4, HSA-CD4 or sMono-CD4, or naive 293 cells were used to make organoids. Organoids were surgically implanted into the peritoneal cavity of 6-week male Swiss (nu/nu) nude mice (Iffa-Credo, France) anaesthetised with Tribromoethanol (ACROS ORGANICS, New Jersey, USA) at 125 mg/kg. Neo-organ formation, implantation, and removal were performed as previously described [15].

All animal studies were performed at the animal facility of Centre d'Étude et de Recherche Viro-Immunologiques (C.E.R.V.I.), Hôpital de la Pitié-Salpêtrière, Paris, France. The animals were cared in accordance with local institutional guidelines.

Preparation of the human serum albumin alginate coated beads and bio-encapsulation of cells

The procedure was as described [23] with minor modifications. The initial aqueous phase was prepared by dissolving three components, sodium alginate (1%), propylene glycerol alginate (PGA, 2%) and human serum albumin (HSA, 5%) in 0.9 % NaCl. After 5 minutes of stirring, the homogeneous solution was centrifuged for 5 min at 5000 rpm to eliminate the air bubbles. Twenty eight millions cells were collected in 500 µl of sterile 0.9 % NaCl, then carefully added and re-suspended in 2 ml of the alginate solution. The resulting

solution containing cells was added dropwise to 12.5 ml of 10 % $CaCl_2$, to form beads. After 5 min of magnetic stirring, beads were washed with 0.9 % NaCl, then the transacylation reaction between the ester groups of PGA and the amine group of HSA was initiated in 10 mM NaOH for 5 min under agitation. A membrane was formed around beads, made of a protein directly bound to polysaccharides through amide linkages. The coated beads were then incubated for 5 min in 0.9 % NaCl, pH 7.0 then incubated in cell culture medium. One batch of beads corresponds to approximately 140 beads. In this condition, the cell number in beads, at the beginning of the culture, is theoretically about 200,000 cells/bead.

Mice plasma analysis: transgene expression *in vivo* in Swiss nude mice

Retro-orbital blood was collected from organoids- or the beads-implanted mice with a 100 µl heparinated capillary pipette under anaesthesia with tribromoethanol. The collected blood was centrifuged at 5000 rpm (2700 relative centrifugal force) and the plasma was stored at -20 C immediately. sMulti-CD4, HSA-CD4 or sMono-CD4 were analysed by ELISA and immunoblot on thawed aliquots.

Analysis of BL4 anti-CD4 mAb secretion

BL4 (Immunotech, Marseille, France: Hybridoma Data Bank, BL4 195 RE 1.G) is a monoclonal antibody which specifically reacts with human CD4 [27]. The BL4 hybridoma cells were produced by fusion of the mouse myeloma Sp2/o with spleen lymphocytes from a BALB/c mouse immunised with human peripheral blood T lymphocytes. A monoclonal antibody (IgG2a κ), BL4, is produced.

Sera were collected from the mice that were implanted with the encapsulated BL4 hybridoma cells and analysed for the presence of BL4 anti-CD4 mAb. Two millions human peripheral blood lymphocytes (PBLs) were incubated with 50 µl of different serum at 1:5 or 1:20 in PBS-1% BSA for 30 min at 4°C. After 3 washes, PBLs were then incubated with 50 µl of anti-mouse-FITC antibody at 1:50 in PBS-1% BSA for 30 min at 4°C. After 3 washes, PBLs were analysed on a FACStar Plus apparatus (Beeton Dickinson).

Histological analysis of the human serum albumin alginate coated beads

The cells containing human serum albumin alginate coated beads were recovered from the mice, washed with PBS and fixed with PBS containing 2 % glutaraldehyde for 48 h. Beads were then dehydrated in 70°, 80°, 90°, 95° and 100° ethanol successively and put in propylene oxide (1 h for each stage). Beads were then incubated in 50% propylene oxide/50% EPON (Merck) for 2h, and in EPON overnight. Finally, , beads were put in EPON solution with 1.5 % of the catalyser (2,4,6-Tris(Dimethylaminomethyl)Phenol : DMP30) DMP30 for 72 to 96 h at 60 °C to allow the resin to polymerize. Samples were finally cut using an ultramicrotome and stained with 0.5 % methylene blue (Sigma) and 0.5 % azur blue II (Merck) for observation.

RESULTS

In vitro expression and characterisation of sMulti-CD4, HSA-CD4 and sMono-CD4 by 293 cells

Neomycin resistant clones of 293 cells transfected with CD4-based expression vector together with pMC1neo poly A were isolated by G418 selection. They were grown to confluence, fed with fresh medium without FCS and 24-h culture supernatants were collected and assayed for the recombinant protein secretion by ELISA. We selected clones secreting sMulti-CD4, HSA-CD4 or sMono-CD4 at a rate of, 4.0, 1.1 and 4.2 $\mu g/10^7$ cells/24h, respectively.

To verify the nature of these secreted proteins, western immunoblot analysis was performed using a polyclonal anti-hCD4 rabbit serum [19] (Fig 1C). Unique 44 or 92kDa bands were detected under both reducing or non-reducing conditions with the supernatant of the 293/sCD4 (lanes 2, 6) or 293/HSACD4 (lane 10), respectively. The 92 kDa band could also be detected using a polyclonal anti-HSA serum (lane 12). A unique 50 kDa band corresponding to the molecular weight of monomeric CD4-C4bpα could also be seen under reducing condition with the supernatant of 293/CD4-C4bpα cells (lane 1). Under non-reducing condition, a unique band of large molecular weight (≈ 350 kDa) could be detected (lane 5). These results indicate that the CD4-based recombinant proteins are efficiently secreted by 293 cells as unique soluble products, in a probable heptameric form for CD4-C4bpα.

sMulti-CD4 inhibits HIV infection better than sMono-CD4

To determine whether sMulti-CD4 secreted by 293 cells retains anti-HIV infection activity, culture supernatant of 293/CD4-C4bpα was tested in HIV infection of P4-CCR5 cells *in vitro*. Several dilutions of the culture supernatant of 293/Cl/CD4-C4bpα or 293/Cl/sCD4 were incubated with 100 tissue culture infectious doses (TCID) of HIV-1_{LAI} for 48 h. The 50% inhibitory concentration of sMulti-CD4 was 35 ng/ml, three times better than that of purified recombinant soluble CD4, 100 ng/ml (Fig. 2A).

These results indicate that the sMulti-CD4 and sMono-CD4 protein produced by the 293 cells are functional. On the contrary, the HSA-CD4 recombinant protein showed a poor capacity to block HIV infection (data not shown).

***In vivo* delivery of sMulti-CD4, HSA-CD4 and sMono-CD4 by organoids implanted in nude mice**

Using the 293/CD4-C4bpα, /HSA-CD4 or /sCD4 cells, we generated the organoids made of 10^7 cells. Two organoids were transplanted into the peritoneal cavity of each Swiss nude mice.

Implantation of sMulti-CD4-secreting organoids resulted in high plasma levels of sMulti-CD4 which increased from 5.5 ± 1.4 ng/ml (mean ± S.E.M.) at 2 weeks post-implantation to 2306 ± 716.7 ng/ml when they were sacrificed. With 293/HSA-CD4-organoids, the HSA-CD4 plasma concentrations were 2.6 ± 0.8 ng/ml at 2 weeks post-implantation and raised to 5432 ± 258 ng/ml at 13 weeks post-implantation. In contrast, we could not detect any plasma sMono-CD4 at 2 weeks post-implantation in 293/sCD4

organoids implanted mice, and only 1.1 ± 0.5 ng/ml and up to 173 ± 91.6 ng/ml at 4 and 13 weeks post-implantation, respectively (Fig. 3A).

Starting at 4-5 weeks after transplantation, all nude mice developed tumours which continued to grow until they were sacrificed for examination. All the neo-organs were found to be connected to the mesenteric tissues or to the bowel, and were well vascularized. Organoid weights, which were approximately 0.2 g before transplantation, raised up to 11,4 ± 5.28 g (mean ± S.D, n=5) with 293/CD4C4bpα organoids and 17.53 ± 1.60 g (n=3) for 293/HSA-CD4 (not significantly different), whereas that of 293/sCD4 were significantly smaller (2.44 ± 2.85, n=5, paired T test, $p<0.05$). No signs of metastasic tumours was seen at macroscopic evaluation. Thus 293 cells are tumorogenic in nude mice.

Characterisation of *in vivo*-expressed recombinant proteins

To investigate if the *in vivo*-expressed sMulti-CD4 was still multimeric, we performed western immunoblot with the plasma of a 293/CD4-C4bpα organoids implanted mice (Fig. 3B). The polyclonal anti-hCD4 serum showed a unique high molecular band (lane 5) identical to that observed with the culture supernatant of these same cells (lane 2), and thus corresponding to sMulti-CD4 molecules.

Plasma from HSA-CD4-organoids implanted mice were also examined using western blotting. A polyclonal anti HSA serum revealed the 92 kDa band corresponding to the HSA-CD4 hybrid (lane 6). The polyclonal antibodies (either anti-hCD4 or anti-HSA) also reacted non-specifically with plasma proteins, resulting in a heterogeneous band pattern identical between organoids-implanted (lane 5, 6) and control mouse (lane 4, 7).

Relationship between plasma levels and numbers of secreting cells

Total body weight plotted versus the plasma concentration at various time points showed a good linear correlation (Fig.3C) suggesting that the 293 cells kept secreting recombinant proteins during tumour growth. It also indicates that the plasma concentration is a linear function of the number of secreting cells.

When the animals were sacrificed, implants were removed, weighed, and the cells were dissociated with *collagenase*. They were then cultured *in vitro* with or without G418, and analysed for the secretion of the transgene products. The recovered 293/CD4-C4bpα cells were secreting sMulti-CD4 at the same level with or without G418, 22 % lower than their secretion level before implantation. In contrast, the recovered 293/HSA-CD4 or 293/sCD4 cells retained the secretion capacity they showed before implantation.

Plasma sMulti-CD4 retains its capacity to inhibit HIV infection

We next investigated whether sMulti-CD4 expressed *in vivo* in nude mice retains its anti-HIV activity with an HIV inhibition assay based on using P4-CCR5 cells [26]. These Hela derived cells co-express human CD4 and CXCR5 and are thus sensitive to HIV infection. They are also transduced with a Tat-inducible Lac-Z gene in order to monitor the HIV infection by LacZ expression. The P4-CCR5 cells were infected with 100 TCID of HIV-1_{LAI} in the presence of different dilutions of either the plasma from 293/CD4-C4bp-organoids implanted mice which contains 4 ug/ml of sMulti-CD4, or the plasma of normal nude mice as a negative control (Fig. 2B). After 48h, the enzymatic detection of β-Gal was performed. The

results indicated that *in vivo*-expressed plasma sMulti-CD4 retained an anti-HIV activity similar to that from *in vitro* expressed sMulti-CD4.

***In vitro* secretion of therapeutic proteins from encapsulated 293 cells**

We investigated the secretion of the recombinant proteins after encapsulation of 293 cells in human serum albumin alginate coated beads. Encapsulated 293 cells were placed in culture medium and the secretion rate of sMono-CD4, HSA-CD4 or sMulti-CD4 was analysed by ELISA. At 4 weeks after encapsulation, secretion rates were 3307, 751 and 3 ng/30beads/day, respectively. Previous analysis allowed to determine that 30 beads contain approximately 6 $x10^6$ cells. The three 293 clones secrete sMono-CD4 (44 kDa), HSA-CD4 (92 kDa) and sMulti-CD4 (350 kDa) at 2372, 663 and 2452 ng/24h/$6x10^6$ cells, respectively. Thus, sMono-CD4 and HSA-CD4 freely diffuse through the beads, but sMulti-CD4 didn't.

Xeno-transplantation in immuno-compromised mice: *in vivo* expression of sMono-CD4 and HSA-CD4 from the encapsulated 293 cells in Swiss Nude mice

In order to evaluate if the encapsulated xenogenic293 cells could secrete sMono-CD4 or HSA-CD4 without tumour formation, 30 beads with 293/sCD4 or 293/HSA-CD4 cells were implanted into each mice and the plasma concentration of sMono-CD4 and HSA-CD4 measured over time. The HSA-CD4 plasma concentration reached a plateau of ≈ 300 ng/ml at 4 weeks post-implantation (Fig.4A upper panel) and stayed at this level for more than 16 weeks post-implantation. Plasma sMono-CD4 concentration was 0.5 ng/ml 2 weeks post-implantation and remained the same level during 13 weeks (Fig.4A lower panel).

The HSA-alginate coated beads-implanted mice were sacrificed and examined macroscopically. All beads were found intact in peritoneal cavity and easily recovered. There was no sign of tumour growth of the cells outside beads. In contrast, the identical 293 clones implanted as organoids in nude mice showed tumour formation in all the mice after 4-6 weeks of implantation. Beads were recovered, put in culture medium *in vitro*, and analysed for transgene secretion. The recovered 293/sCD4 and 293/HSA-CD4 cells in the beads were shown to keep secreting their transgene products at 4000 and 400 - 880 ng/30 beads/24h respectively after their recovery, which is about the same rate as those of before implantation.

Transplantation in immuno-competent mice: *in vivo* secretion of BL4 anti-CD4 mAb from the encapsulated BL4-hybridoma cells in Balb/c mice

Sera from BALB/C mice implanted with the BL4-hybridoma cells containing human serum albumin alginate beads were collected at various times after the implantation, and analysed. The results showed that serum BL4 anti-CD4 mAb concentration continuously increased during the 30 days of follow up when the beads were left in place (Fig. 4B lower panel). In contrast, when the beads were explanted after two weeks, BL4 anti-CD4 mAb secretion gradually decreased (Fig.4B upper panel).

Mice were sacrificed, then beads were recovered, put in culture medium *in vitro*, and subsequent BL4 anti-CD4 mAb secretion was verified (data not shown).

Histological analysis of the beads implanted in mice

Beads harbouring either allogenic or xenogenic cells were explanted from either nude or immuno-competent mice and analysed. 293/sCD4 or 293/HSA-CD4 containing beads

recovered nude mice 16 weeks after implantation remained intact (Fig.5A), with fibrosis at the surface (Fig.5B), and viable cells clustered at the center of the beads (Fig.5C).

The 293/sCD4 containing beads explanted from immuno-competent BALB/c mice were still closed (Fig.5D) but their outer membrane was altered by an extensive immune response from the host with many lymphocytes as well as phagocytes, which digested the surface of the membrane (Fig. 5E). The 293 cells appeared severely affected as well.

In contrast, the beads containing encapsulated BL4 hybridoma cells implanted in Balb/c mice remained intact at least until 4 weeks post-implantation (Fig.5F), surrounded by fibrosis and neo-vascularisation despite a wall originating from a xenogenic human serum albumin (Fig.5G). The host immune response against the beads was moderate and BL4 hybridoma cells appeared healthy. Viable cells were seen at the center of the beads (Fig.5H).

DISCUSSION

Parameters affecting the plasma concentration of therapeutic protein secreted by genetically modified cells:

Many diseases may benefit from *in vivo* delivery of therapeutic proteins by genetically modified cells. The first question in regard to the feasibility of such approaches could be whether the therapeutic level of the protein is likely to be achieved with the methods envisioned. These levels are likely to depend on the secretion capacity and the number of the implanted genetically modified cells, but they also depend on the pharmacokinetic properties of the protein. The treatment of HIV infection with CD4-based soluble inhibitors offers a good paradigm to study various strategies to produce these recombinant therapeutic proteins by implantation of genetically modified cells. Indeed, it has long been shown that a soluble form of the HIV receptor (sMono-CD4) could efficiently neutralise culture-adapted HIV isolates *in vitro*. However, because of its quick *in vivo* clearance (45 minute in human), the high dose of sMono-CD4 (3 to 10 mg) needs to be injected every 8 hour in humans to maintain therapeutic concentrations [28]. It prompted the development of CD4-based recombinant molecules that were designed to have better pharmacokinetic properties, as well as improved efficacy. Such molecules are still under development as purified recombinant proteins to be injected to patients. They should be useful for obtaining high plasma concentration for a short period of time. However, for chronic diseases like HIV infection, another treatment modality such as the *in vivo* delivery of CD4 derived protein by genetically modified cells can be envisioned for a long term delivery. Using stable 293 cells secreting 3 different CD4-based proteins with different half-life, we generated organoids. We observed

that plasma half-life is a major parameter that determine plasma concentrations. In addition, taking advantage of the fact that organoids made of 293 cells are tumourigenic in nude mice, we also showed that there was a linear relationship between the number of the secreting cells and the plasma concentrations that raised up to 125 µg/ml with HSA-CD4. Thus, the number of secreting cells and the plasma half life of the secreted recombinant protein are indeed two main parameters for obtaining high plasma concentrations.

sMulti-CD4, a potentially clinically useful inhibitor of HIV infection

Soluble CD4 (sMono-CD4), has been shown to neutralise a wide range of culture adapted and primary HIV isolates in vitro [25], [29], [30], [31], [32], [33], [34] by preventing virus from binding to its receptor [35] and also shown to inhibit cell-to-cell transmission. Clinical trials have shown that purified sMono-CD4 has no significant clinical or immunologicaltoxicity and induced a significant decline of the viral load [28],[16]. However, because of its rapid *in vivo* clearance (45 min-1 h in human), a large dose of sMono-CD4 (3 to 10 mg) was needed to be injected every 8 h to maintain plasma concentrations at therapeutic level [28]. IgG-CD4 fusion protein has also been developed, which has a significantly prolonged plasma half-life [30]. It also appears to have a better efficacy for neutralising HIV due to their di- or tetra-valence [17]. We have already shown that a minimal C4bp C-terminal α-fragment derived from a naturally heptameric molecule is sufficient to obtain soluble heptameric fusion protein [22]. Multimers are assembled in the cell without necessity for secondary modifications, resulting in the secretion of a unique, covalently linked soluble molecule, although the expression vector codes only for the monomer. We used the same strategy to produce a soluble multimeric multivalent CD4, sMulti-CD4, in order to have a

longer plasma half-life and a better antiviral activity than those of sMono-CD4. We indeed report here that sMulti-CD4 is approximately 3 time more efficient than sMono-CD4 for neutralising HIV infection. In addition, although we have not determined it precisely, the plasma half life of sMulti-CD4 should be significantly longer than that of sMono-CD4, being compatible with an *in vivo* use. Finally, sMulti-CD4 has a good physicochemical stability *in vivo* as shown by immunoblotting as well as the antiviral effect of *in-vivo* expressed sMulti-CD4. Altogether, these results warrant further development of this molecule for HIV treatment, notably using gene therapy.

Serum albumin alginate beads coated with cross-linked bio-polymer as a tool for encapsulating genetically modified cells

The use of organoid to secret recombinant protein suffers from some difficulties. Indeed, because genetically modified cells are to be re-implanted into a recipient, they need to be autologous to prevent their rejection by the host immune responses. Therefore, they are usually derived from autologous skin fibroblasts which needs to be transduced and then expended before re-implantation. This is a labour intensive and costly procedure which has to be tailored to every patient. The use of an « universal » cell line expressing the protein of interest to be re-implanted in any patient would obviously much simplify the therapeutic procedures.

However, such allogenic or xenogenic cells need to be protected from the host immune response. Many encapsulation process have been developed to achieve this goal [12, 14, 36-38]. Here we have used human serum albumin alginate coated beads to encapsulate human or mouse cells [23]. This method has the advantages of generating large beads that are quite solid, that can be easily manipulated and that contain significant number of cells. After encapsulation of genetically modified 293 cells, we detected expected levels of sMono-CD4 and HSA-CD4,

but only very low levels of sMulti-CD4 which is the product of CD4-C4bpα gene. When we encapsulated the hybridoma cells secreting an IgG, the antibody was secreted from the beads. Therefore, the molecular cut off for this type of beads should be between 150 and 350 kDa.

When the encapsulated cells were implanted in immuno-deficient nude mice, xenogenic cells survived and secreted the recombinant protein for a long period of time (Fig. 5A, B, C). Interestingly, although 293 cells are tumourigenic in nude mice, no tumour could be detected, indicating that the coated alginate beads could protect the host from the tumourigenic cells within the beads.

When the similar encapsulated cells were implanted in normal Balb/c mice, the xenogenic cells (293 cells) were rapidly eliminated (Fig.5D, E), most probably due to an antibody mediated immune response. However, when allogenic cells (BL4) were re-implanted in immuno-competent animals (Balb/c mice), they survived and secreted the recombinant protein (Fig.5F ,G, H). Altogether, these results demonstrate that the coated-alginate beads can protect allogenic but not xenogenic cells from immune rejection. If such results can be confirmed in primates, this should be useful for a clinical use, which is likely to rely on allogenic cells rather than xenogenic ones.

Usually, therapeutic proteins need to be delivered under strict control to avoid over expression and possible side effects. There are several promoters available which could be controlled *in vivo* such as a system using tetracycline [39] or rapamycin [40]. Efficient long-term gene transfer into muscle tissue for immuno-competent mice by adeno-associated virus (AAV) vector has been reported [41], [42], [43], [44], [45], [46]. It is also reported that control of gene expression in vivo after gene transfer using AAV vector is possible [6, 47]. Although AAV are still difficult to produce under GMP, this may represent a more practical

method than the use of encapsulated cells. However, the latter method has two potential advantages: First, it does not require to inject a recombinant viral vector that always carries the risk of dissemination; Second, implanted beads can always be explanted to stop recombinant protein production if necessary. This might provide an important safety control when the over expression of a therapeutic protein can trigger severe side effects.

In summary, allogeniccells encapsulated in calcium-alginatebeads coated with cross-linked bio-polymer appear a promising system for the in vivo delivery of therapeutic proteins, includingimmunoglobulins.

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Fig. 1. Expression in 293 cells

A) Expression plasmids: pCI/sCD4, pCI/HSA-CD4 and pCI/CD4-C4bpα plasmids coding for human sMono-CD4, HSA-CD4 and sMulti-CD4 respectively.

B) Schematic presentation of CD4-based proteins

sCD4 consists of 4 domains. CD4-C4bpα is a hybrid gene resulting in the association between sCD4 and C4bpα genes that allow the secretion of sCD4 homo-multimeric molecules. HSA-CD4 is a hybrid of human serum albumin (HSA) merged to the first two domains of human CD4.

C) Expression of sMulti-CD4, sMono-CD4, and HSA-CD4 by 293 cells *in vitro*

Immunoblot analysis of culture supernatants, as revealed by a polyclonal serum directed against human CD4 under reducing (lanes 1 - 4), non-reducing (lanes 5 - 8); Supernatants of the 293 cells secreting sMulti-CD4 (lanes 1, 5), and those of soluble CD4 (lanes 2 and 6); diluted purified soluble CD4 (lanes 3 and 7) as positive control; supernatants of untransfected 293 cells (lanes 4 and 8)..

Polyclonal serum against human serum albumin (lanes 10 and 11) and polyclonal serum directed against human CD4 (lanes 12 and 13) revealed 92 kDa bands corresponding to HSA-CD4 in the supernatants of 293/CI/HSA-CD4 (lanes 10 and 12); supernatants of untransfected 293 cells (lanes 11 and 13).

Fig. 2

Antiviral activity of sMulti-CD4

A) sMulti-CD4 by 293 cells *in vitro*: P4-CCR5 indicator cells were infected with 100 Tissue Culture infectious doses of $HIV_{1\,AI}$ with different concentrations of sMulti-CD4 or sMono-

CD4 secreted by 293 cells *in vitro* for 48h, then enzymatic detection of beta-gal was performed. Results represent the percentage of the β-gal compared with that of β-gal of P4-CCR5 cells infected with HIV_{LAI} without any sMono-CD4 or its derivatives.

B) sMulti-CD4 expressed *in vivo* : P4-CCR5 cells were infected with HIV_{LAI} with different dilutions of the plasma of the nude mouse implanted with 293/Cl/CD4-C4bpα-organoids.

As a negative control, we infected the P4-CCR5 cells with HIV_{LAI} in the presence of the supernatant of naive 293 cells and it showed no significant effect on HIV infection at less than 2.5 %. But at more than 7.5%, normal plasma also showed antiviral activity as those of Multi-CD4 or sCD4.

Fig.3

***In vivo* expression of sMulti-CD4, HSA-CD4 and sMono-CD4 by organoids in nude mice:**

A) Plasma level of sMulti-CD4, HSA-CD4 and sMono-CD4 as a function of time after implantation of the organoids. Swiss nude mice were implanted either with the 293/Cl/CD4-C4bpα-organoids (n= 6), the 293/Cl/HSA-CD4-organoids (n=3) or the 293/Cl/sCD4-organoids (n=6), then bled at the indicated number of weeks after the implantation and the plasma was stored frozen. The expression of those molecules was determined by ELISA and is presented as mean ± S.E.M.

B) Immunoblot analysis of the plasma using a polyclonal serum directed against human CD4 under non-reducing condition: lane 1, molecular weight marker; lane 2, supernatants of a 293 cells secreting sMulti-CD4; lane 3, supernatants of the 293 cells secreting sCD4; lane 4, 1:2

diluted plasma from untreated Swiss nude mice; lane 5, 1:2 diluted plasma from a 293/CI/CD4-C4bpα-organoids implanted nude mice.

Immunoblot with a polyclonal rabbit against HSA under reducing condition in lane 6 and 7. Lane 6, 1:50 diluted plasma from a 293/CI/HSA-CD4-organoids implanted nude mice; lane 7, 1:50 diluted plasma from an untreated Swiss nude mice.

C) Significant correlation between body weight (BW) and the plasma concentration: BW and plasma concentration of sMulti-CD4 ,HSA-CD4 or sMono-CD4 in organoids implanted mice at each time point were plotted. Significant correlation between them was shown (sMulti-CD4, r^2=0,989; HSA-CD4, r^2=0,991; sMono-CD4, r^2= 0,946). Data shown are from a representative animal of each experimental group.

Fig.4

A) *In vivo* expression of HSA-CD4 and sMono-CD4 encapsulated in human serum albumin alginate coated beads in nude mice:

Swiss nude mice were implanted either with the encapsulated 293/CI/HSA-CD4 (upper panel) or 293/CI/sCD4 cells (lower panel), then bled at the indicated number of weeks after the implantation. The plasma concentration of those molecules was determined by ELISA and is presented as mean ± S.E.M.

B) *In vivo* expression of BL4 mAb in BALB/c mice:

Upper panel: The encapsulated BL4 hybridoma cells were implanted in 2 of BALB/c mice for 2 weeks, then the beads were explanted. During 2 weeks of implantation, fluorescence intensity continued to increase and it started to decrease after their explantation. Lower panel: In a mouse, the beads were left implanted in a Balb/c mouse for 30 days. The Fluorescence intensity continued to increase until 30 days.

Fig.5

Encapsulated xenogenic cells in Nude mice

The coated alginate beads containing 293/CI/sCD4 cells recovered from a Swiss Nude mouse 66 days post-implantation. **A, B) :** No major degradation of the membrane by the host cells, but only the fibrosis was seen around the bead. **C) :** In the center of the bead, clusters of the 293 cells alive ,were seen. Bars : A) 500 µm; B) 200 µm; C) 100 µm.

Encapsulated xenogenic cells in Balb/c mice

D, E) : Human serum albumin alginate coated beads containing 293/CI/sCD4 cells recovered from a Balb/c mouse one month after implantation. The membrane of the beads were strongly affected by the host phagocytes to develop fibrosis and vascularization around the beads. The 293 cells in beads show the morphological characteristics of dead cells. Bars : D) 1 mm; E) 100 µm.

Xenogenic beads in Balb/c mice

Human serum albumin alginate coated beads containing BL4 hybridoma cells, removed from a Balb/c mice 1 month after implantation. **F, G) :** The membrane of the beads did not show any major deterioration and remained intact. Only slight fibrosis was seen around the beads. **H) :** In the center of the beads, BL4 hybridoma cells were alive.

Bar indicates in F) 1 mm, G) 100 µm and H) 50 µm respectively.

References

1. Hortelano G, Al-Hendy A, Ofosu FA, Chang PL. Delivery of human factor IX in mice by encapsulated recombinant myoblasts: a novel approach towards allogeneic gene therapy of hemophilia B. *Blood* 1996;87:5095

2. Al-Hendy A, Hortelano G, Tannenbaum GS, Chang PL. Correction of the growth defect in dwarf mice with nonautologous microencapsulated myoblasts--an alternate approach to somatic gene therapy. *Hum Gene Ther* 1995;6:165

3. Moullier P, Marechal V, Danos O, Heard JM. Continuous systemic secretion of a lysosomal enzyme by genetically modified mouse skin fibroblasts. *Transplantation* 1993;56:427

4. Moullier P, Bohl D, Heard JM, Danos O. Correction of lysosomal storage in the liver and spleen of MPS VII mice by implantation of genetically modified skin fibroblasts [see comments]. *Nat Genet* 1993;4:154

5. Moullier P, Bohl D, Cardoso J, Heard JM, Danos O. Long-term delivery of a lysosomal enzyme by genetically modified fibroblasts in dogs. *Nat Med* 1995;1:353

6. Bohl D, Salvetti A, Moullier P, Heard JM. Control of Erythropoietin Delivery by Doxycycline in Mice After Intramuscular Injection of Adeno-Associated vector. *Blood* 1998;92:1512

7. Leroy-Viard K, Rouyer-Fessard P, Beuzard Y. Improvement of mouse beta-thalassemia by recombinant human erythropoietin. *Blood* 1991;78:1596

8. Villeval J, Rouyer-Fessard P, Blumenfeld N, Henri A. Retrovirus-mediated transfe of the erythropoietin gene in hematopoietic cells improves murine beta thalassemia. *Blood* 1994;84:928

9. Olivieri N, Freedman M, Perrine S, Dover G, Sheridan B. Trial of recombinant erythropoietin: three patients with thalassemia intermedia. *Blood* 1992;80:3258

10. Rachmilewitz E, Goldfarb A, Dover G. Administration of erythropoietin to patients with beta-thalassemia intermedia: A preliminary trial. *Blood* 1988;80:1145

11. Nisli G, Kavakli K, Aydinok Y, Oztop S, Cetingul N and Basak N. Recombinant erythropoietin trial in children with transfusion-dependent homozygous beta-thalassemia. *Acta Haematol* 1997;98:199

12. Chang PL, Van Raamsdonk JM, Hortelano G, Barsoum SC, MacDonald NC and Stockley TL. The in vivo delivery of heterologous proteins by microencapsulated recombinant cells. *Trends Biotechnol* 1999;17:78

13. Tai IT, Sun AM. Microencapsulation of recombinant cells: a new delivery system for gene therapy. *Faseb J* 1993;7:1061

14. Lanza RP, Chick WL. Immunoisolation: at a turning point. *Immunology Today* 1997;18:135

15. Valere T, Bohl D, Klatzmann D, Danos O, Sonigo P and Heard JM. Continuous secretion of human soluble CD4 in mice transplanted with genetically modified cells. *Gene Ther* 1995;2:197

16. Kahn JO, Allan JD, Hodges TL, Kaplan LD, Arri CJ, Fitch HF, Izu AE, Mordenti J, Sherwin JE, Groopman JE and et al. The safety and pharmacokinetics of recombinant soluble CD4 (rCD4) in subjects with the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. A phase I study [see comments]. *Ann Intern Med* 1990;112:254

17. Capon DJ, Chamow SM, Mordenti J, Marsters SA, Gregory T, Mitsuya H, Byrn RA, Lucas C, Wurm FM, Groopman JE and et al. Designing CD4 immunoadhesins for AIDS therapy. *Nature* 1989;337:525

18. Traunecker A, Schneider J, Kiefer H and Karjalainen K. Highly efficient neutralization of HIV with recombinant CD4-immunoglobulin molecules. *Nature* 1989;339:68

19. Idziorek T, Klatzmann D. Construction of CD4-Based Chimeric Molecules by Chemical Cross-Linking. *AIDS Res. Hum. Retroviruses* 1991;7:529

20. Yeh P, Landais D, Lemaitre M, Maury I, Crenne JY, Becquart J, Murry-Brelier A, Boucher F, Montay G, Fleer R and et al. Design of yeast-secreted albumin derivatives for human therapy: biological and antiviral properties of a serum albumin-CD4 genetic conjugate. *Proc Natl Acad Sci U S A* 1992;89:1904

21. Berkower I, Mostowski H, Bull TE and Murphy D. CD4-IgG binding threshold for inactivation of human immunodeficiency virus type 1. *J Inf Dis* 1996;173:863

22. Libyh MT, Goossens D, Oudin S, Gupta N, Dervillez X, Juszczak G, Cornillet P, Bougy F, Reveil B, Philbert F, Tabary T, Klatzmann D, Rouger P and Cohen JHM. A Recombinant Human scFv Anti-Rh(D) Antibody With Multiple Valences Using a C-Terminal Fragment of C4-Binding Protein. *Blood* 1997;90:3978

23. Levy MC, Edwards-Levy F. Coating alginate beads with cross-linked biopolymers: a novel method based on a transacylation reaction. *J Microencapsul* 1996;13:169

24. D'Souza MP, Geyer SJ, Hanson CV, Hendry RM, Milman CI. Evaluation of monoclonal antibodies to HIV-1 envelope by neutralization and binding assays: an international collaboration. *AIDS* 1994;8:169

25. Deen KC, McDougal JS, Inacker R, Folena-Wasserman G, Arthos J, Rosenberg J, Maddon PJ, Axel R and Sweet RW. A soluble form of CD4 (T4) protein inhibits AIDS virus infection. *Nature* 1988;331:82

26. Charneau P, Alizon M, Clavel F. A Second Origin of DNA Plus-Strand Synthesis Is Required for Optimal Human Immunodeficiency Virus Replication. *J. Virol.* 1992;66:2814

27. Taylor J, Wiliams A, Morten J, Morten H. Analysis of CD4 monoclonal antibodies using human x mouse hybrid cell-lines OKT4. In: A. McMichael and e. al., eds. Analysis of CD4 monoclonal antibodies using human x mouse hybrid cell-lines OKT4. 1987;234

28. Schooley RT, Merigan TC, Gaut P, Hirsch MS, Holodniy M, Flynn T, Liu S, Bylington RE, Henochowicz S, Gubish E and et al. Recombinant soluble CD4 therapy in patients with the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. A phase I-II escalating dosage trial. *Ann Intern Med* 1990;112:247

29. Clapham PR, Weber JN, Whitby D, McIntosh K, Dalgleish AG, Maddon PJ, Deen KC, Sweet RW and Weiss RA. Soluble CD4 blocks the infectivity of diverse strains of HIV and SIV for T cells and monocytes but not for brain and muscle cells. *Nature* 1989;337:368

30. Byrn RA, Mordenti J, Lucas C, Smith D, Marsters SA, Johnson JS, Cossum P, Chamow SM, Wurm FM, Gregory T and et al. Biologicalproperties of a CD4 immunoadhesin. *Nature* 1990;344:667

31. Fisher RA, Bertonis JM, Meier W, Johnson VA, Costopoulos DS, Liu T, Tizard R, Walker BD, Hirsch MS, Schooley RT and et al. HIV infection is blocked in vitro by recombinant soluble CD4. *Nature* 1988;331:76

32. Hussey RE, Richardson NE, Kowalski M, Brown NR, Chang HC, Siliciano RF, Dorfman T, Walker B, Sodroski J and Reinherz EL. A soluble CD4 protein selectively inhibits HIV replication and syncytium formation. *Nature* 1988;331:78

33. Traunecker A, Luke WKarjalainen K. Soluble CD4 molecules neutralize human immunodeficiency virus type 1. *Nature* 1988;331:84

34. Daar ES, Li XL, Moudgil T, Ho DD. High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates. *Proc Natl Acad Sci USA* 1990;87:6574

35. Moore JP, McKeating JA, Norton WA, Sattentau A. Direct measurement of soluble CD4 binding to human immunodeficiency virus type 1 virions: gp120 dissociation and its implications for virus-cell binding and fusion reactions and their neutralization by soluble CD4. *J.Virol.* 1991;65:1133

36. Joly A, Desjardins JF, Fremond B, Desille M, Campion JP, Malledant Y, Lebreton Y, Semana G, Edwards-Levy F, Levy MC and Clement B. Survival, proliferation, and functions of porcine hepatocytes encapsulated in coated alginate beads: a step toward a reliable bioartificial liver. *Transplantation* 1997;63:795

37. Brauker J, Frost GH, Dwarki V, Nijjar T, Chin R, Carr-Brendel V, Jasunas C, Hodgett D, Stone W, Cohen LK and Johnson RC. Sustained expression of high levels of human factor IX from human cells implanted within an immunoisolation device into athymic rodents. *Hum Gene Ther* 1998;9:879

38. Serguera C, Bohl D, Rolland E, Prevost P, Heard JM. Control of Erythropoietin Secretion by Doxycycline or Mifepristone in Mice Bearing Polymer-Encapsulated Engineered Cells. *Hum. Gene Ther.* 1999;10:375

39. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W and Bujard H. Transcriptional activation by tetracyclines in mammalian cells. *Science* 1995;268:1766

40. Magari SR, Rivera VM, Iuliucci JD, Gilman M, Cerasoli F, Jr. Pharmacologic control of a humanized gene therapy system implanted into nude mice. *J Clin Invest* 1997;100:2865

41. Xiao X, Li J, Samulski RJ. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J Virol* 1996;70:8098

42. Kessler PD, Podsakoff GM, Chen X, McQuiston SA, Colosi PC, Matelis LA, Kurtzman GJ and Byrne BJ. Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. *Proc Natl Acad Sci U S A* 1996;93:14082

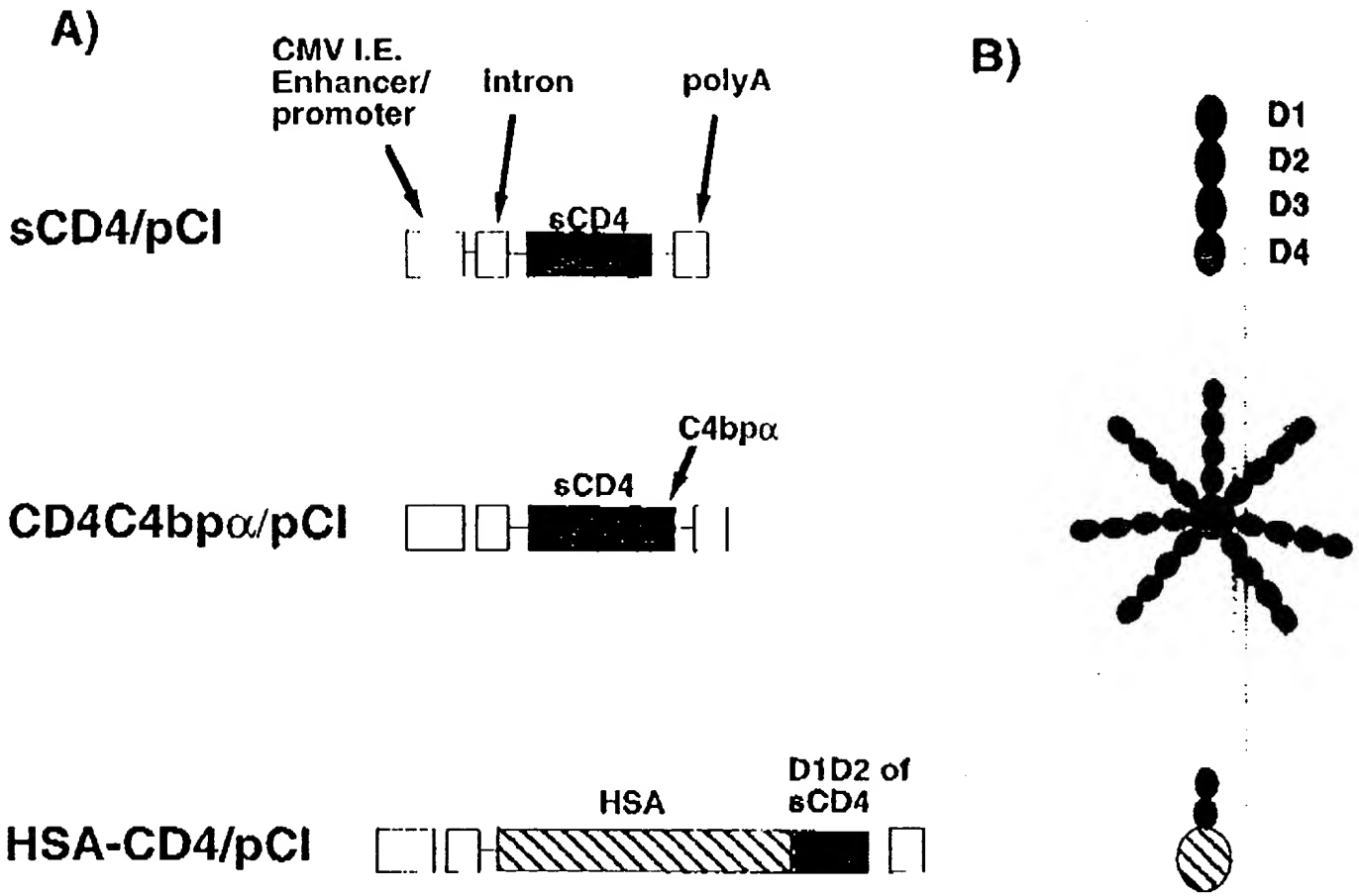
43. Herzog RW, Hagstrom JN, Kung SH, Tai SJ, Wilson JM, Fisher KJ and High KA. Stable gene transfer and expression of human blood coagulation factor IX after intramuscular injection of recombinant adeno-associated virus. *Proc Natl Acad Sci U S A* 1997;94:5804

44. Snyder RO, Spratt SK, Lagarde C, Bohl D, Kaspar B, Sloan B, Cohen LK and Danos O. Efficient and stable adeno-associated virus-mediated transduction in the skeletal muscle of adult immunocompetent mice. *Hum Gene Ther* 1997;8:1891

45. Clark KR, Sferra TJ, Johnson PR. Recombinant adeno-associated viral vectors mediate long-term transgene expression in muscle. *Hum Gene Ther* 1997;8:659

46. Murphy JE, Zhou S, Giese K, Williams LT, Escobedo JA and Dwarki VJ. Long-term correction of obesity and diabetes in genetically obese mice by a single intramuscular injection of recombinant adeno-associated virus encoding mouse leptin. *Proc Natl Acad Sci U S A* 1997;94:13921

47. Rendahl KG, Leff SE, Otten GR, Spratt SK, Bohl D, Van Roey M, Donahue BA, Cohen LK, Mandel RJ, Danos O and Snyder RO. Regulation of gene expression in vivo following transduction by two separate rAAV vectors. *Nat Biotechnol* 1998;16:757


A)
CMV I.E.
Enhancer/
promoter
Intron
polyA
sCD4/pCI
sCD4
B)
D1
D2
D3
D4
C4bpα
CD4C4bpα/pCI
sCD4
D1D2 of
sCD4
HSA
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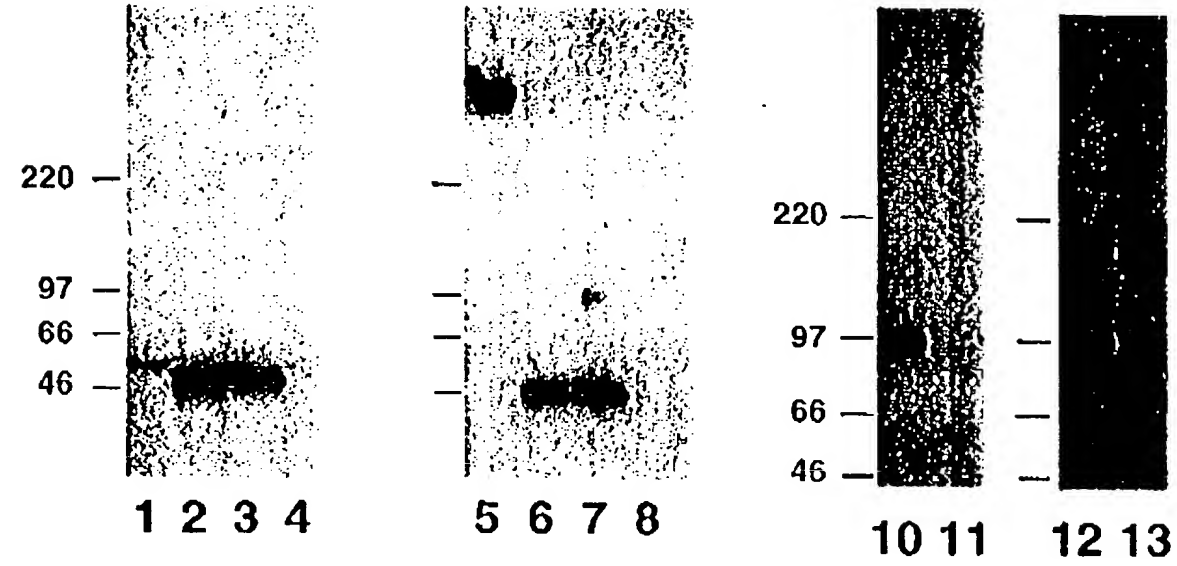
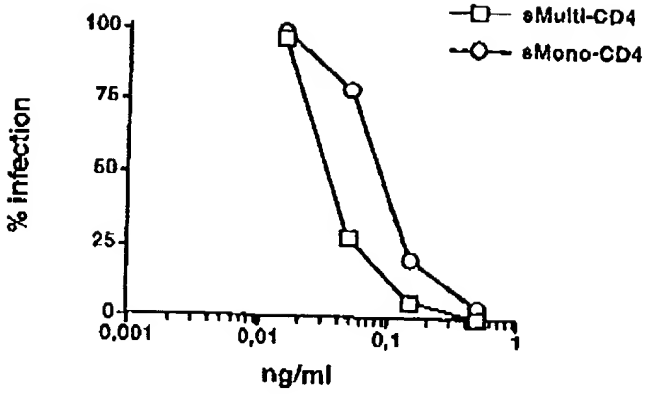

C)
220
97
66
46
1 2 3 4
5 6 7 8
220
97
66
46
10 11
12 13

Figure 1

A)


sMulti-CD4
sMono-CD4
% infection
100
75
50
25
0
0,001
0,01
0,1
1
ng/ml

B)

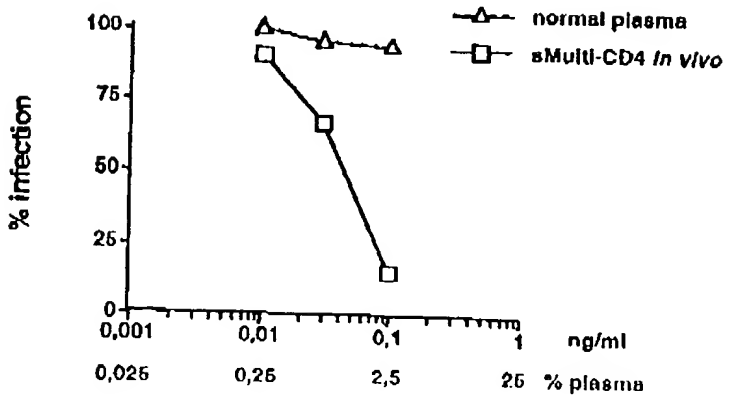

normal plasma
sMulti-CD4 *in vivo*
% infection
100
75
50
25
0
0,001
0,01
0,1
1
ng/ml
0,025
0,25
2,5
25
% plasma

Figure 2

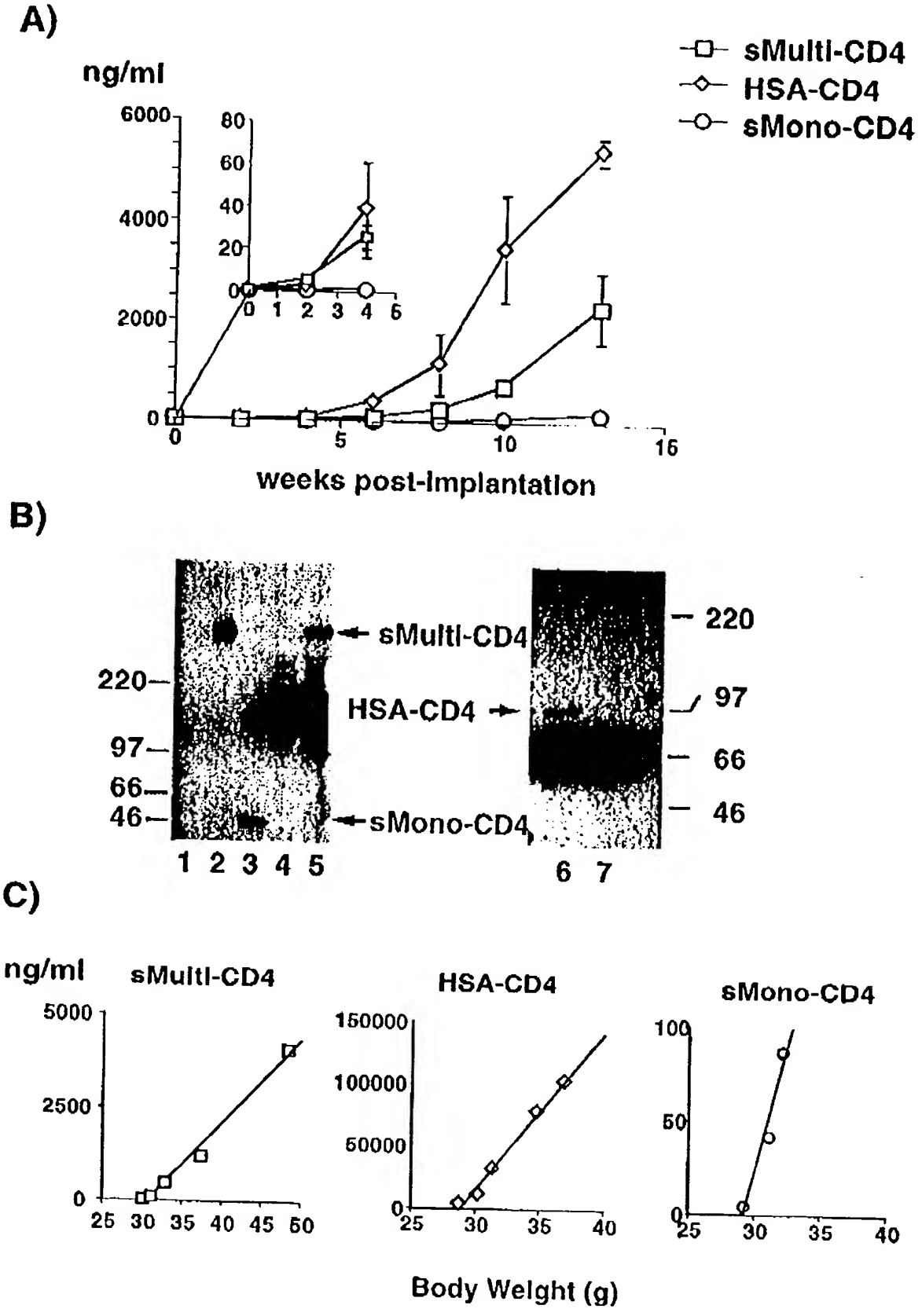
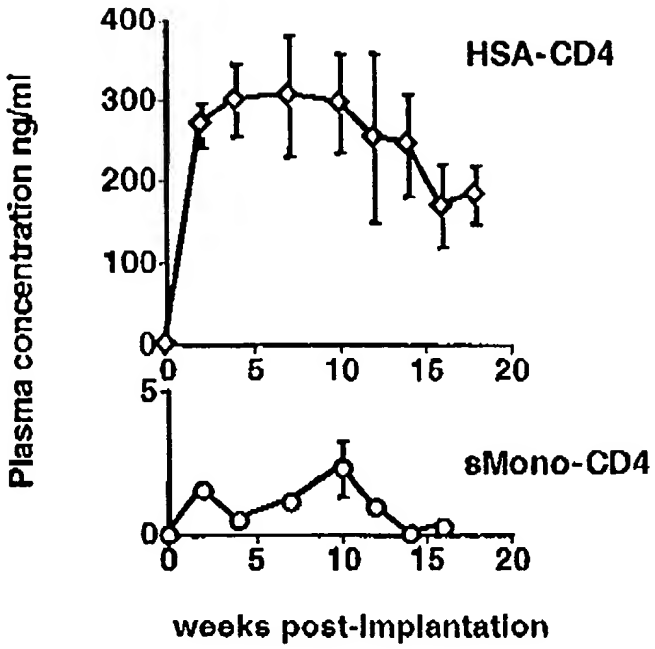
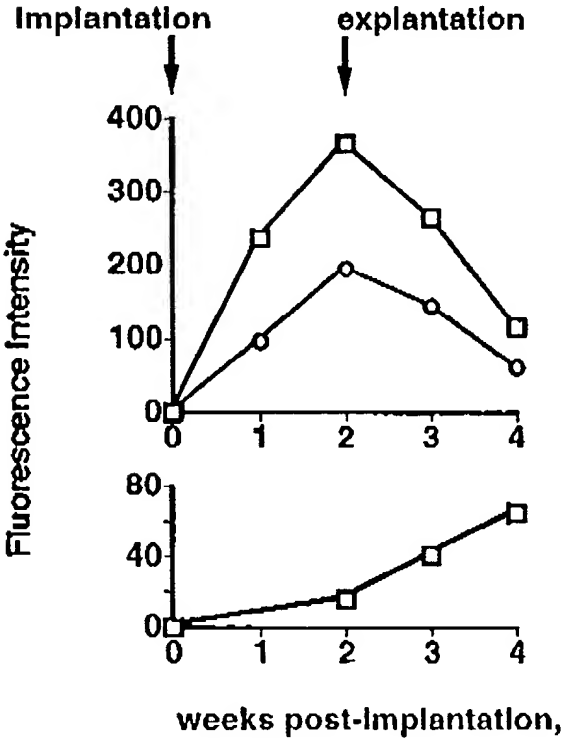

A)
ng/ml
sMulti-CD4
HSA-CD4
sMono-CD4
6000
4000
2000
0
80
60
40
20
0
0 1 2 3 4 5
0
5
10
15
weeks post-Implantation
B)
sMulti-CD4
HSA-CD4
sMono-CD4
220
97
66
46
1 2 3 4 5
6 7
C)
ng/ml
sMulti-CD4
HSA-CD4
sMono-CD4
5000
2500
0
25 30 35 40 45 50
150000
100000
50000
0
25 30 35 40
100
50
0
25 30 35 40
Body Weight (g)

Figure 3

A)


Plasma concentration ng/ml
400
300
200
100
0
HSA-CD4
0
5
10
15
20
5
0
sMono-CD4
0
5
10
15
20
weeks post-Implantation

B)


Implantation
explantation
Fluorescence Intensity
400
300
200
100
0
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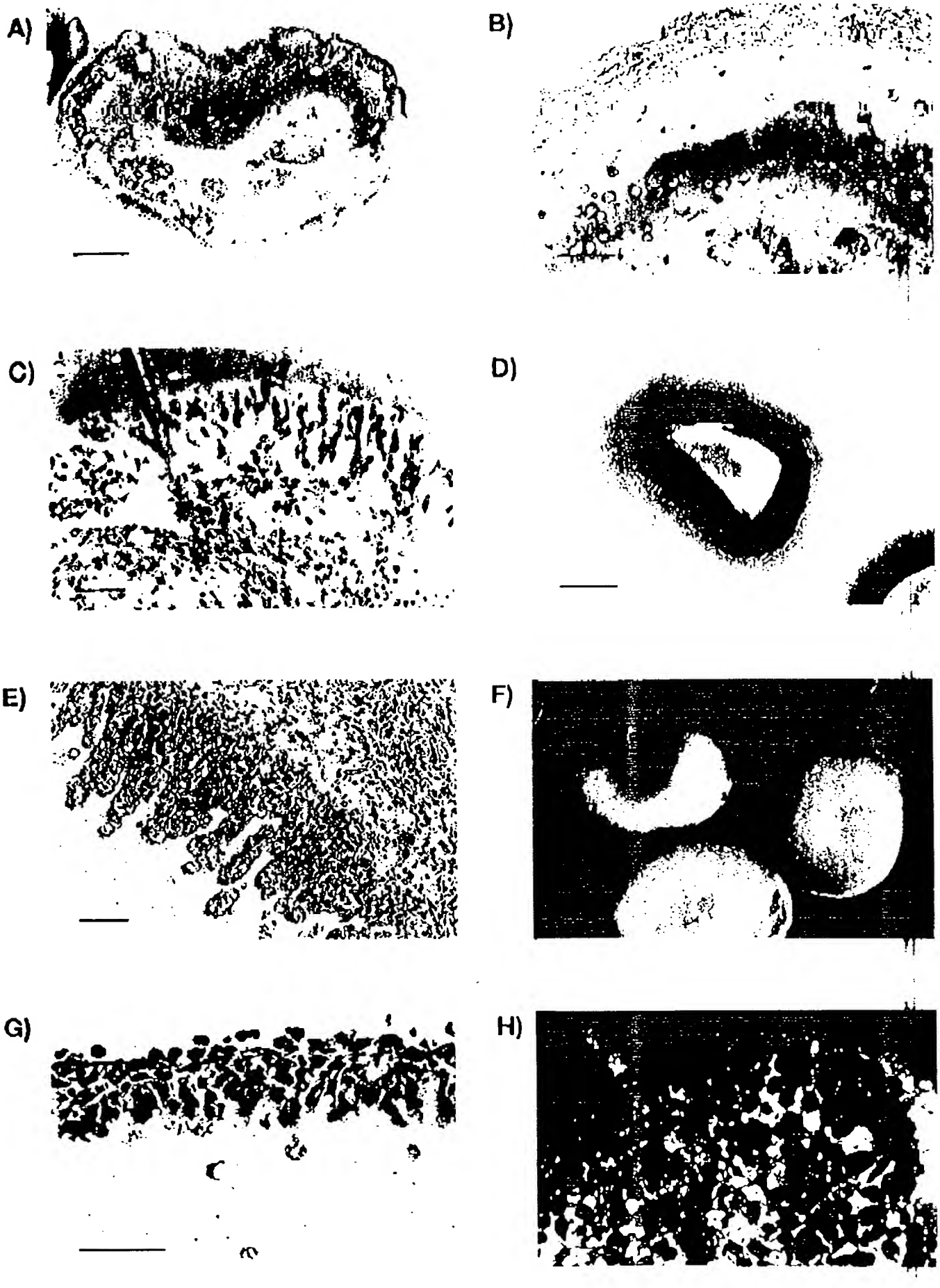
1
2
3
4
80
40
0
0
1
2
3
4
weeks post-Implantation,

Figure 4


A)
B)
C)
D)
E)
F)
G)
H)